Body protein reserves sustained maternal performances in early lactation but dietary protein was necessary to maintain performance and immune responses to Nippostrongylus brasiliensis in lactating rats

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1. Title

a. Body protein reserves sustained maternal performances in early lactation but dietary protein was necessary to maintain performance and immune responses to *Nippostrongylus brasiliensis* in lactating rats

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(ii) Abbreviation used: ALOX15, arachidonate 15-lipoxygenase; CP, crude protein; DM, dry matter; FEC, fecal egg counts; GI, gastrointestinal; IGD, initial gestation diet; IL, interleukin; PPAR, peroxisome proliferator activated receptor; PPRI, periparturient relaxation of immunity; Th1, T helper type 1; Th2, T helper type 2.

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2. Abstract

Background: It has been shown that dietary protein supplementation during lactation boosts immunity in *Nippostrongylus brasiliensis*-infected periparturient rats. It is not known whether body protein reserves accumulated during gestation have a similar effect during lactation.

Objective: This study aimed to quantify the impact of body protein reserves and dietary protein supplementation on maternal performances and immune responses to *N. brasiliensis* during lactation.

Methods: Multiparous female Sprague-Dawley rats were given a primary infection of *N. brasiliensis* prior to mating and restrictedly fed either 60 g (Lge) or 210 g (Hge) crude protein (CP) per kg dry matter (DM) until parturition. Parturition onwards, dams were restrictedly fed either 100 g (Lla) or 300 g (Hla) CP per kg DM, generating 4 different dietary treatments. A subset of rats was sampled before parturition; post-parturition, dams were secondary infected with *N. brasiliensis* and samples were collected at day 5 and 11 post parturition.

Results: Maternal performance until parturition, as measured by pup weight, was better in Hge rats compared to Lge (Lge 4.84 g, Hge 6.15 g, S.E.D. 0.19). On day 11, pup weight of dams with reduced protein reserves receiving protein during lactation (Lge-Hla, 20.28 g) was higher compared to their counterparts from Hge-Lla dams (17.88 g, S.E.D. 0.92). Worm counts were significantly different between Lge-Lla (253; 95% CI 124 to 382) and Hge-Hla fed dams (87; 95% CI 22 to 104) on day 11 (P=0.024). Expression of splenic *Il13* and *Alox15* was significantly higher (P<0.05) in Hge-Hla dams compared to Lge-Lla on day 5.

Conclusions: Although protein reserves were adequate to maintain maternal performances in the early stage of lactation in dams infected with *N. brasiliensis*. they were not adequate to maintain maternal performances and effective immune responses at the later stages. Dietary protein supplementation was required to achieve this.

Keywords: Periparturient relaxation of immunity; Gastrointestinal nematodes; *Nippostrongylus brasiliensis*; Nutrition; Dietary protein; Lactation

3. Introduction

Periparturient relaxation of immunity (PPRI) in mammals, a phenomenon where expression of acquired immunity breaks down during late pregnancy and subsequent lactation, plays a crucial part in epidemiology of gastrointestinal (GI) nematode parasitism (1, 2). It has been hypothesized that PPRI may reflect a preferential partitioning of scarce nutrients, particularly protein, to reproductive effort rather than to immune functions (2). A number of studies in small ruminants have demonstrated that protein supplementation during lactation at times of protein scarcity results in decreased PPRI as demonstrated by lower fecal nematode egg counts (FEC), worm burdens, and higher number of immune cells (3).

This nutritional basis of PPRI has also been assessed in rodent models, particularly during a secondary infection with *Nippostrongylus brasiliensis* in the lactating rat (4, 5, 6). In rats, the first half of gestation is an anabolic phase when animals accumulate protein reserves, and the second half of gestation is a catabolic phase when animals withdraw the accumulated protein to support the rapid growth of their fetus (7). Feeding a low protein diet during the second half of gestation depleted dam’s protein reserves significantly (8), with dams subsequently manifesting PPRI against *N. brasiliensis*

infection (9). On the other hand, protein supplementation during the second half of gestation resulted in dams having additional body protein reserves at parturition compared to those fed a low protein diet (8). It may be expected that this additional endogenous protein reserves reduce the impact of dietary protein scarcity and improve the immune responses to *N. brasiliensis* in the subsequent lactation period.

The aim of this experiment was to evaluate the effect of dietary protein supplementation and body protein reserves on maternal performances and immune responses in periparturient rats. This study is advancing the understanding of nutritional mechanisms not only in animals but also in human populations at times of extreme nutrient demand. Our hypothesis was that increased body protein reserves accumulated during gestation and dietary protein supplementation during lactation independently improve host maternal performances, reduce parasitism and enhance immune responses to secondary *N. brasiliensis* infection at times of protein scarcity.

4. Methods

Experimental animals

The experiment described below was approved by Scotland's Rural College's ethical review committee (ED 35/2013) and carried out under Home Office authorization (PPL60/4395). One hundred and four multiparous female Sprague Dawley rats were used (Charles River UK Ltd, UK). Rats were housed individually in solid bottom cages, with fresh sawdust provided weekly. For mating purposes, each female was placed in a wire-bottomed cage with a proven male breeder overnight and mating was confirmed through the presence of the vaginal plug (defined as day −22). Plastic bubble wrapping material was provided on the day before expected parturition date for nesting. The parturition date

was defined as the morning when parturition was observed to have finished and is described as day 0 from here onwards.

Infection protocol

Rats were infected subcutaneously in the hind limb with 1600 third-stage infective larvae of *N. brasiliensis* in 0.5 mL sterile PBS according to our previously established protocol (9). All rats were given primary infection at least 14 days prior to mating. Due to the restricted number of the male breeder, average period between primary infection and when mating was confirmed was 25 days. Feces collection and FEC were performed as described previously (9, 10). On day 2 post parturition dams were given a secondary infection of 1600 third stage larvae, defined as an infected group, or sham infected with 0.5mL sterilized PBS, defined as a control group.

Feeding protocol and experimental design

Rats had free access to fresh water throughout the experiment. From the primary infection and until mating was confirmed, rats were fed a standard rat chow diet (Standard RM3; Moisture 10%, Crude oil 4.20%, Crude protein 22.45%, Crude fibre 4.42%, Ash 8.05%, Nitrogen free extract 50.4%; Special Diets Services, UK). Once the mating was confirmed (day −22), rats were allocated to feeding treatments as explained below on the basis of body weight (**Figure 1**). Mated rats were fed an initial gestation diet (IGD) containing 210 g crude protein (CP) per kg dry matter (DM) *ad libitum* for the first 10 days of gestation. During the second half of gestation, rats went under restricted feeding with iso-energetic diets calculated to supply either 60 g CP per kg DM (Lge diet), or 210 g CP per kg DM (Hge diet) until parturition. Restricted feeding was achieved by offering the mean daily DM intake of individual rat observed between day −16 and day

−12. On day −2, 13 rats were euthanized to assess carcass composition and evaluate the accumulation of body protein reserves in Lge (*n*=6) and Hge (*n*=7) animals and feeding treatment effects on maternal performances and baseline immune functions. From parturition onwards the remaining dams were fed iso-energetic diets, calculated to supply 100 g CP per kg DM (Lla) or 300 g CP per kg DM (Hla), creating effectively 4 different dietary treatments through gestation and lactation, i.e. Lge-Lla, Lge-Hla, Hge-Lla, and Hge-Hla. Dams were fed restrictedly at 90% of DM intake of similar diets in previous experiments under *ad libitum* feeding (4, 5), where dam parturition body weight was used as a scaling factor. Food allowances increased as the experiment progressed post parturition, reflecting the natural increase in food intake in lactating dams as observed in the previous experiments (4, 5). The composition and chemical analysis of the diets are seen in **Supplemental Table 1**. A total of 49 dams were euthanized on day 5 (3 days post secondary infection or sham-infection) and 27 dams on day 11 (9 days post secondary infection) of the experiment. On sampling, animals were sedated through increasing CO_2 inhalation, and then euthanized by CO_2 asphyxiation. Pups were euthanized by cervical dislocation. Data were collected from 89 rats; 15 rats had to be removed from the experiment either because they did not conceive, or culled for ethical reasons, such as pupping trauma. As a consequence, actual sample size for each group ranged from *n*=6 to 8 for infected rats and *n*=4 to 6 for sham-infected controls

Measurements

Body weight and food intake Rats were weighed daily throughout the experiment. DM intake was assessed daily by weighing food offered and refused. Pup number was

standardized to 12 on day 1 of lactation, and pups were bulk-weighed daily until day 5 or 11.

Carcass analysis After removing all organs, carcasses were weighed, and maintained in −20 °C until analysis. Carcasses were freeze dried (Edwards Freeze Dryer Super Modulyo, Edwards Vacuum, UK) at −40 °C and pressure at −108 Torr. The completion of the process was confirmed by reaching the point of no further weight loss. Carcasses were then milled using a food processor (Magimix, UK) and analyzed for crude protein and fat content.

Worm burdens, colon egg counts and ELISA for serum antibodies Worm burdens and colon egg counts were determined as previously described (9, 10). For ELISA, 96 well polystyrene microplates (Nunc, Denmark) were coated with 2 μg/mL of purified mouse ani-rat IgE (553914, BD Biosciences, UK) or 5 μg/mL of *N. brasiliensis* adult worm antigen in carbonate buffer for total IgE and specific IgGs respectively and were incubated overnight. Serum samples were diluted at 1:10 and 1:100 for IgE, at 1:100 for IgG1 and IgG2b, and at 1:500 for IgG2a. Standards for IgE were prepared by a 2-fold dilution of commercially available IgE (PRP07A, AbD serotec, UK) starting from 10 μg/mL. Several samples from the final endpoint were pooled and a 2-fold dilutions were prepared as standards for IgGs. Following the addition of the samples and standards to the wells, plates were incubated overnight at 4 °C and washed. Biotin conjugated mouse anti-rat antibodies were diluted in 1% BSA/PBS to 1 μg/mL for IgE, IgG1, IgG2b (553916, 553890, 553898, BD Biosciences, UK), and 0.5 μg/mL for IgG2a (553894, BD Biosciences, UK). After incubation, plates were washed and peroxidase labelled streptavidin (1:1000, KPL, Inc., USA) was added. After incubation and washing, TMB

peroxidase substrate (KPL, Inc., USA) was added and the reaction was stopped by adding 0.18M of H_2SO_4. The absorbance at 450 nm was measured with a microplate reader.

RNA extraction/cDNA synthesis At post mortem, the spleen and a 1 cm cut small intestine sample taken at a 25 cm distance from pylorus were placed in RNA later (Sigma, UK). RNA extraction was performed using the Qiagen RNA extraction kit (Qiagen, UK) and cDNA synthesis was conducted using Thermo Scientific Verso™ cDNA synthesis kits (Thermo Scientific, UK) following the manufacturer's guideline.

Real time quantitative PCR Primers were selected either from previously published papers (11, 12, 13, 14, 15) or designed with the online Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Supplemental Table 2**, Invitrogen, UK). The genes of target selected were associated with either T helper type 2 (Th2) immune response or type 1 (Th1) response. For the Th2 response, expressions of interleukin (IL)-4 (*Il4*) and IL-13 (*Il13*), and arginase 1 (*Arg1*) as a marker for alternatively activated macrophages, were determined (16). For Th1, the expressions of interferon γ (*Ifng*) and tumor necrosis factor α (*Tnfa*), and inducible nitric oxide synthase (*Nos2*) as a marker for classically activated macrophages, were determined (17). Additional target genes included i) intracellular adhesion molecule 1 (*Icam1*), which governs leucocyte recruitment during inflammation (18) and ii) peroxisome proliferator activated receptor (PPAR)-γ (*Pparg*) and arachidonate 15-lipoxygenase (ALOX15, *Alox15*) as anti-inflammatory molecules induced by IL-4 (19, 20, 21). PCR was performed on a MX300P QPCR system (Agilent Technologies, UK) with Brilliant Ultra-Fast 2×SYBR® green QPCR Master Mix (Agilent Technologies, UK). Standard curves were generated by purified real time PCR products using Wizard SV gel and a PCR clean

up system kit (Promega, UK). The thermal profile of the reaction consisted of 95 °C for 3 min, 40 cycles of 95 °C for 20 s, and annealing at 60 °C for 20 s. Real time PCR was performed with a melting curve analysis to establish the purity of each amplified product. All PCR products were sequenced to confirm that the correct products were amplified. Two reference genes, *Ywhaz* and *Actb*, were selected by the *Rattus norvegicus* 6 gene geNorm kit (PrimerDesign Ltd, UK) and Qbase plus software (Biogazelle BE, Belgium). Their geometric mean was used to normalize expression values of the target genes.

Statistical analysis

All generated data were checked for normality and Log (n+1) transformations were performed for serum immunoglobulin, colon egg counts and worm burden to stabilize the variance before statistical analysis. Those transformed data are reported as back transformed means with back transformed lower and upper limits of transformed error bars.

Gestation: To investigate the effects of the gestation diet (Lge vs Hge) on performance and baseline immune responses (serum antibodies and gene expressions) in the absence of an active nematode infection, a one-way ANOVA was performed in samples recovered on day −2. Gene expression analysis in the spleen was analyzed by the non-parametric Mann-Whitney U test due to non-normally distributed data despite the transformation.

Lactation: 1) To investigate the effect of time, dietary treatments and their interaction on immunological parameters during an active parasite infection, an ANOVA through a 2 (gestation diet) × 2 (lactation diet) × 2 (time points) factorial design was performed, where main effects and interactions were tested. 2) To investigate the effects

of parasitism (control and infected), dietary treatments and their interaction on immunological parameters, an ANOVA through a 2 (gestation diets) × 2 (lactation diets) × 2 (level of parasitism) factorial design was performed. Values for gene expression for spleen and intestine on both endpoints were analyzed using Mann-Whitney U test due to non-normally distributed data. 3) Parasitological parameters, i.e. Colon egg counts and worm burdens were analyzed in a 2 (Lge or Hge) × 2 (Lla or Hla) factorial ANOVA to test the effect of gestation and lactation diets on these variables. Colon egg counts were only analyzed on day 11 (no eggs were present on day 5); worm counts were expected to be higher on day 11 due to the life cycle of the nematode, so time was not included in the statistical model.

Maternal performances, i.e. dam weight, food intake and mean pup weight were additionally assessed through repeated measures ANOVA to quantify the temporal variation of the treatments (22). To quantify the effect of gestation and lactation diets, carcass characteristics were analyzed in 2 (Lge or Hge) × 2 (Lla or Hla) factorial ANOVA. Covariate adjustment using dam body weight on day −22 was included for dam body weights, carcass weight, spleen and pup weight analyses. The average food intake (DM/g/day) from day −16 to −12 of gestation, when all the animals were fed the IGD diet, was included as a covariate for food intake analysis. All statistical analyses were carried out using GenStat 16th edition (VSN international, UK). *P* values <0.05 were considered statistically significant; *P* values between 0.05 and 0.1 are reported as tendencies. Fisher's Least Significant Difference was used to identify the significant interactions between the different groups.

5. Results

Feeding a high protein diet during the second half of gestation improves performance and selected baseline immune response in pregnant rats

There was a significant diet × days interaction on both DM intake (P<0.05) and dam weight (P<0.05) during gestation with Lge rats showing a reduction in their DM intake and weight gain as they approached parturition compared to Hge rats (**Figure 2A and 2B**). Although litter size was not affected by the dietary treatments (P>0.1, data not shown), mean fetus weight was significantly affected by gestation diet, which was heavier in Hge rats compared to Lge (P=0.001; **Table 1**). Carcass weight (P=0.041), carcass protein (P=0.017) and spleen weight (P=0.052) were all heavier in Hge rats compared to Lge (**Table 1**).

In the absence of an active nematode challenge, serum IgG1 of Hge rats tended to be greater than that of Lge rats (P=0.082), whereas other immunoglobulins, IgE, IgG2a and IgG2b, were not affected by the gestation diet (P>0.1, data not shown). Hge rats had significantly higher expression of Th2 immune response related genes, *Il4*, *Arg1*, *Alox15*, *Pparg*, and Th1 related, *Nos2* and *Ifng*, measured in the spleen than Lge rats (P<0.05; **Figure 3A**). In contrast, Lge rats had higher expression of intestinal *Icam1* than Hge rats (P<0.05; **Figure 3B**).

Body protein reserves accumulated during the second half of gestation improves maternal performances during lactation

The difference in dam weight created during gestation between Lge and Hge rats was maintained until day 11, with Lge-Lla and Lge-Hla rats weighing less than Hge-Lla and Hge-Hla rats during lactation (P<0.001, **Figure 4A**). There was a significant interaction between lactation diet and days, where Lge-Lla and Hge-Lla dams showed lower weight

gain compared to Lge-Hla and Hge-Hla dams (P<0.001, **Figure 4A**). There was a significant lactation diet × days effect on DM intake, with Hla dams having greater DM intake compared to Lla dams as the experiment progressed (P<0.001, **Figure 4B**). Mean pup weight was significantly heavier in Hge rats compared to Lge rats at parturition (Lge 4.84 ± SE 0.12 g, Hge 6.15 ± 0.15 g, S.E.D. 0.19). However, lactation diet started showing significant effect on mean pup weight from day 3 of lactation (P=0.014), and this effect of lactation diet surpassed gestation diet on day 11 where pups of dams with reduced protein reserve but protein supplemented during lactation (Lge-Hla) outweighed their counterparts, i.e. Hge-Lla dams (Lge-Hla 20.28 ± SE 0.63 g, Hge-Lla 17.88 ± 0.63 g, S.E.D. 0.92, **Figure 4C**).

Carcass analysis was carried out on samples collected at the end of the experiment to evaluate how this was affected by the gestation and lactation diets. Carcass protein was affected by both gestation and lactation diet on day 11 (P=0.009 and P<0.001 respectively, **Table 2**), whereas carcass weight and fat were affected only by gestation diet (P<0.001, **Table 2**).

Body protein reserves accumulated during the second half of gestation does not affect parasitological and immune responses during an active* N. brasiliensis *infection

The effect of gestation and lactation diet were not significant for colon egg counts at day 11 and worm numbers recovered at day 5 (P>0.1, **Figure 5A and 5B**). However, there was a significant difference in the worm counts recovered from Lge-Lla (253; 95% CI 124 to 382) and Hge-Hla dams (87; 95% CI 22 to 104) compared at day 11 (P=0.024; **Figure 5B**). Therefore, gene expression analysis was carried out in samples recovered from dams in these two treatments. Hge-Hla dams showed significantly higher mRNA

253 expression of *Alox15* and *Ifng* than Lge-Lla dams on day 5 (P<0.05; **Figure 5C**). Dietary
254 treatments did not affect expression of target genes on day 11 (data not shown).

255 Gestation and lactation diets significantly affected spleen weight, which was the
256 highest for Hge-Hla dams at both time points (**Figure 6A**). Spleen weight of infected
257 dams was significantly heavier than that of the control ones (P=0.003). Dams showed
258 heavier spleens on day 5 compared to day 11, irrespective of the dietary treatment
259 (P<0.001). Gene expression analysis was only conducted on samples originated from
260 infected Lge-Lla and Hge-Hla dams as they showed the greatest difference in spleen
261 weight. *Il13* and *Alox15* expression was significantly higher (P<0.05) in Hge-Hla dams
262 on day 5 (**Figure 6B**). However, none of the genes examined on day 11 were affected by
263 the dietary treatments (data not shown).

264 Gestation diet did not affect any of the antibody levels measured. IgG1 levels were
265 affected by the lactation diet × infection status interaction (P=0.028) where re-infection
266 increased antibody levels in Lla but not in Hla dams. There was also a significant
267 lactation diet × time points interaction on IgG1 (P=0.045) and IgG2a (P=0.027) levels,
268 where Hla dams showed higher antibody levels on day 11 compared to Lla dams. No
269 significant treatment effects were observed on serum IgG2b level. There was a significant
270 difference between time points for IgE levels, which were higher on day 11 compared to
271 day 5 (P=0.002); no other significant effects were observed for IgE. (**Figure 7**)

272 **6. Discussion**

273 Dietary protein supplementation during lactation has been shown to enhance immunity in
274 *N. brasiliensis* re-infected lactating rat model (4, 5, 6), but the role of body protein
275 reserves during the same time has not been previously assessed. Here, we investigated for

the first time whether protein reserves accumulated during gestation can sustain both maternal performances and immune responses during the subsequent lactation period. Our data showed that protein reserves accumulated during gestation were adequate to maintain dam and pup weight in the early stage of lactation, but not in the later stage. Likewise, protein reserves accumulated during gestation improved baseline immunity in the absence of an infection, but they were not adequate to sustain effective immune responses during an active *N. brasiliensis* infection.

Gestation Feeding Hge diet during gestation resulted in improved overall maternal performances compared to Lge; Hge rats maintained significantly heavier body weights compared to Lge throughout the latter half of gestation. Body composition analysis showed that this was attributed to an increase in body protein and not body fat, which is consistent with a previous study (8). Although Lge rats used up their accumulated protein reserve from the anabolic phase of gestation to maintain fetus growth, this was still penalized in these rats; on the other hand, rats on the Hge diet maintained their body reserves and supported fetus growth.

These improved maternal performances in rats of the Hge diet here may also be because Hge rats maintained their intake during lactation at a higher level than Lge rats. The evidence is consistent with previous studies where rats showed suppression in feed intake when offered a low protein diet (4, 5, 8, 9, 23). The reason for the difference in feed intake of rats in the two dietary treatments could be attributed to the lower protein to carbohydrate ratio in the Lge diet compared to Hge. Whilst the Lge rats may have aimed to increase protein intake, this would have resulted in an increase in the carbohydrate intake. In non-reproducing rats, carbohydrate excess is taken up by white and brown

adipose tissues, metabolized and stored (24). However, during late pregnancy and lactation, animals alter their adipose tissue metabolism resulting in decreased ability for carbohydrate uptake (24). As Pine *et al*. (8) suggested, the protein to carbohydrate ratio in diets with low protein content may create unbalanced carbohydrate metabolism, because of these animals' inability to properly metabolise carbohydrate excess; this may be responsible for the reduced feed intake observed in Lge rats.

Although protein supplementation during gestation did not affect serum immunoglobulin levels, expression of splenic *Il4*, *Arg1*, *Pparg*, and *Alox15*, which encodes effector molecules related to Th2 immunity, was higher in Hge rats than in Lge rats on day −2. IL-4 is a major cytokine of Th2 immunity and induces alternatively activated macrophage, which one of its signature enzyme marker is Arginase 1 (16). PPAR-γ has shown to promote macrophage differentiation to alternatively activated macrophage (20), and also ALOX15 is reported to generate ligands for activating PPAR-γ in IL-4 dependent manner (21). Hence, our data suggest that dietary protein supplementation during gestation promoted splenic Th2 responses that persisted even after worms were expelled from the host. Although splenic cell numbers were not quantified here, spleen weight was heavier in Hge rats, which implies that the differential gene expression between Lge and Hge rats may be associated with the number of splenic T cells, B cells and macrophages. Indeed, short-term protein malnutrition has been reported to cause a reduction in both T and B cells in the spleen (25), suggesting that protein scarcity could down-regulate immune function as a whole rather than selectively impairing specific T cell lineage. In our study, the upregulation of the expression of

additional genes in Hge rats, such as *Ifng* and *Nos2*, which are Th1 related, may offer some support of this suggestion.

In conclusion, we have shown for the first time that protein supplementation during the second half of gestation improves performance and baseline immune response just before parturition, even in the absence of an active nematode infection.

Lactation Consistently with what happened during gestation, the lactation diet affected maternal performances; with Lge-Hla and Hge-Hla dams outperforming Lge-Lla and Hge-Lla dams by day 11. Dams on Hge-Lla diet were able to maintain their weight and support the pup growth to the same level as Lge-Hla dams for the first 8 days of lactation. However, loss of body protein reserve appeared to be significant in these dams and pup weight of Lge-Hla dams eventually surpassed that of Hge-Lla, suggesting that available protein reserve may have been utilised by day 8. Although rats usually show significant increase in feed intake during lactation, which is regulated by neural activity and hormonal secretion (26, 27), the imbalanced protein: carbohydrate ratio in Lla diet (8) as discussed previously may have prevented them from increasing their intake, which resulted in negative maternal performances during lactation.

Although carcass analysis did not show significant difference in body fat levels on day −2, rats on the Lge diet in gestation showed a significantly lower body fat level compared to Hge rats on day 11. Previous evidence has shown that the body fat accumulated during gestation is mobilized during lactation and the rate of fat loss is the same irrespective of the diet offered during this period (23). Therefore, the difference in body fat observed in Lge vs Hge rats on day 11, may be attributed to the significant reduction in DM intake observed from day −4 until parturition, hence reduction in overall

daily energy intake in Lge rats (Lge 0.37 ± SE 0.02 MJ, Hge 0.45 ± 0.01 MJ, S.E.D. 0.011, gestation diet × days $P<0.001$). One could argue that this difference in fat reserves may have an effect on immune responses. However, as previous studies have provided evidence that the breakdown of immunity to parasite infection is sensitive to protein rather than to energy scarcity (28, 29), it is unlikely that the variation in body fat levels will have impacted on the degree of PPRI as observed in this study.

Our data support previous evidence where protein supplementation resulted in reduced worm numbers in lactating rats. We have shown that these effects were mediated at systemic level; protein supplementation resulted in higher levels of serum IgG1 and IgG2a (**Figure 7**), an observation is in agreement with previous evidence, where increased protein supply during lactation tended to result in higher levels of serum total IgG (6). Such immunoglobulins originate from the spleen, which is orchestrating the systemic immune responses during nematode infection and producing Th2 cells (30). Although gestation and lactation diets both had a significant effect on spleen weight, Lge-Hla dams showed heavier spleen weight compared to Hge-Lla dams, indicating that dietary protein may have a bigger impact in spleen weight than body protein reserve. An increase in the spleen size has been associated with active immune response, specifically an increase in hemopoietic activity and supply of immune effector cells; splenomegaly has been observed in mice during a *N. brasiliensis* (31) and a *Heligmosomoides polygyrus bakeri* infection (32). This increase in spleen size was smaller in protein deficient mice compared to those on a protein sufficient diet (32). Gene expression analysis from the spleen revealed that expression of *Il13*, a Th2 cytokine, and *Alox15* was significantly higher in Hge-Hla compared to Lge-Lla dams. Similarly, when a protein

deficient diet was offered in *H. polygyrus bakeri* infected mice, IL-4 secretion from spleen cells was significantly lower compared to mice offered a protein sufficient diet (33). These and our data indicate that protein deficiency penalizes expression of Th2 cytokines systemically, which results in persistent nematode infection.

In summary, difference in the level of dietary protein fed during pregnancy and lactation induced significant metabolic changes and immune responses against a gastrointestinal nematode infection in periparturient rats. Although protein reserves accumulated over gestation were utilized to maintain maternal performances in the early stage of lactation, diet became more important as a source of protein to maintain performance and immune responses in the later stages of lactation. The immune responses tested here were significantly improved when animals were offered high protein throughout gestation and into subsequent lactation period, indicating the importance of both historic and current protein intake. The evidence provided in this study highlights the role of protein from both body reserves and diet in improving maternal performance and immunity against nematode infection at times of extreme protein demand which could assist towards the development of sustainable parasite control strategies in mammals.

7. Acknowledgements

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389 primary responsibility for final content. All authors read, added critical comments and
390 approved the final manuscript.

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Table 1. Carcass weight, carcass protein, carcass fat, spleen weight and fetus weight on day −2 for rats offered Lge or Hge diet[1]

Diet	Carcass weight (g)	Carcass protein (g/carcass)	Carcass fat (g/carcass)	Spleen weight (g)	Fetus weight (g)
Lge (*n*=6)	140.4 ± 6.97	55.12 ± 1.96	67.21 ± 5.92	0.62 ± 0.05	3.12 ± 0.10
Hge (*n*=7)	162.6 ± 6.45	62.72 ± 1.81	80.21 ± 5.48	0.79 ± 0.05	3.69 ± 0.09
S.E.D.	9.506	2.675	8.072	0.074	0.136
Effect of diet[2]	*P*=0.041	*P*=0.017	NS	*P*=0.052	*P*=0.001

[1]Values are mean ± SEs. Covariate adjustment using dam body weight on day −22 was made.

[2]An one-way ANOVA is performed and *P* values <0.05 were considered statistically significant. Exact *P* values are given when value is below 0.1; *P*>0.1 is considered to be not significant (NS).

Table 2. Carcass weight, carcass protein and carcass fat on day 11 for dams offered either Lge-Lla, Lge-Hla, Hge-Lla and Hge-Hla diet[1]

Diet	Carcass weight (g)	Carcass protein (g/carcass)	Carcass fat (g/carcass)
Lge-Lla (*n*=7)	86.55 ± 4.04	49.86 ± 1.26	22.48 ± 3.48
Lge-Hla (*n*=7)	89.47 ± 3.87	53.16 ± 1.21	19.03 ± 3.33
Hge-Lla (*n*=7)	107.13 ± 3.88	52.29 ± 1.21	37.58 ± 3.34
Hge-Hla (*n*=6)	106.54 ± 4.21	58.89 ± 1.32	31.15 ± 3.63
S.E.D	5.687	1.776	4.899
Gestation diet[2]	*P*<0.001	*P*=0.009	*P*<0.001
Lactation diet[2]	NS	*P*<0.001	NS
Gestation × Lactation diet[2]	NS	NS	NS

[1]Values are mean ± SEs. Covariate adjustment using dam body weight on day −22 was made.

[2]2 (Gestation diet) × 2 (Lactation diet) ANOVA is performed and *P* values <0.05 were considered statistically significant. Exact *P* values are given when value is below 0.1; *P*>0.1 is considered to be not significant (NS).

Figure 1. Feeding and infection protocol. Rats were fed restrictedly either 60 g (Lge), or 210 g CP per kg DM (Hge) during second half of gestation. From parturition onwards the remaining dams were fed 100 g (Lla) or 300 g CP per kg DM (Hla), creating effectively 4 different dietary treatments, i.e. Lge-Lla, Lge-Hla, Hge-Lla, and Hge-Hla. CP, crude protein; DM, dry matter; IGD, initial gestation diet; L3, third stage larvae.

Figure 2. Body weight (A) and DM intake (B) of the rats offered either Lge or Hge during second half of gestation. Repeated measures ANOVA was performed to investigate the effect of gestation diet over time. Values are means ± SEs, Lge *n*=6, Hge *n*=7.

Figure 3. Gene expression analysis for spleen (A) and intestine (B) from rats offered either Lge or Hge on day −2. Mann-Whitney U test was performed for spleen, whereas one-way ANOVA was performed to test the effect of Lge and Hge diet for intestine. Values were normalized using reference genes *Actb* and *Ywhaz*, and result for each gene is shown in relative quantity against Lge diet. Values for spleen are median (IQRs), for intestine mean ± SEs. Lge *n*=6, Hge *n*=7. * Significant difference between Lge and Hge, $P<0.05$.

Figure 4. Body weight (A), DM intake (B) and pup weight (C) on day 11 of dams offered either Lge-Lla, Lge-Hla, Hge-Lla or Hge-Hla diet. Repeated measures ANOVA was performed to investigate the effect of gestation and lactation over time. Secondary infection of *N. brasiliensis* on day 2 is marked by an arrow. Values are means ± SEs. Lge-Lla *n*=7, Lge-Hla *n*=7, Hge-Lla *n*=7 and Hge-Hla *n*=6.

Figure 5. Colon egg count (A) and worm burden (B) on day 5 and 11 of dams offered either Lge-Lla, Lge-Hla, Hge-Lla or Hge-Hla diet and gene expression in the intestine on day 5 of dams offered either Lge-Lla or Hge-Hla diet (C). Parasitological responses were analyzed by an ANOVA through 2 (gestation diet) × 2 (lactation diet) factorial design for each day 5 and 11. Log (n+1) transformations were performed prior to analysis for all the data. Values are backtransformed mean ± backtransformed lower and upper limits of transformed error bars. Gene expressions were analyzed by Mann-Whitney U test. Values were normalized using *Actb* and *Ywhaz*, and result for each gene is shown in relative quantity against Lge-Lla diet. Values are median (IQRs). *n*=Day 5, Day 11; Lge-Lla *n*=8, 7 Lge-Hla *n*=7, 7 Hge-Lla *n*=6, 7 and Hge-Hla *n*=7, 6. * Significant difference between Lge-Lla and Hge-Hla, $P<0.05$.

Figure 6. Spleen weight on day 5 and 11 of dams offered either Lge-Lla, Lge-Hla, Hge-Lla or Hge-Hla diet (A) and splenic gene expressions on day 5 of dams offered either Lge-Lla or Hge-Hla (B). The main effect and interactions of (1) parasitic status and diet, and (2) dietary treatments and time points of spleen weight were analyzed in a 2 × 2 × 2 ANOVA. Covariate adjustment using dam body weight on day −22 was made.

Values are means ± SEs. Gene expressions were analyzed by Mann-Whitney U test. Values were normalized using *Actb* and *Ywhaz*, and result for each gene is shown in relative quantity against Lge-Lla diet. Values are median (IQRs). *n*=Day 5 control, Day 5, Day 11; Lge-Lla *n*=5, 8, 7 Lge-Hla *n*=6, 7, 7 Hge-Lla *n*=4, 6, 7 and Hge-Hla *n*=6, 7, 6. * Significant difference between Lge-Lla and Hge-Hla, $P<0.05$.

Figure 7. Serum IgE (A), IgG1 (B), IgG2a (C) and IgG2b (D) level on day 5 and 11 of dams offered either Lge-Lla, Lge-Hla, Hge-Lla or Hge-Hla diet. The main effect and interactions of (1) parasitic status and diet, and (2) dietary treatments and time points were analyzed in a 2 × 2 × 2 ANOVA. Log (n+1) transformations were performed prior to analysis for all the data. Values are backtransformed mean ± backtransformed lower and upper limits of transformed error bars. *n*=Day 5 control, Day 5, Day 11; Lge-Lla *n*=5, 8, 7 Lge-Hla *n*=6, 7, 7 Hge-Lla *n*=4, 6, 7 and Hge-Hla *n*=6, 7, 6.

Figure 1

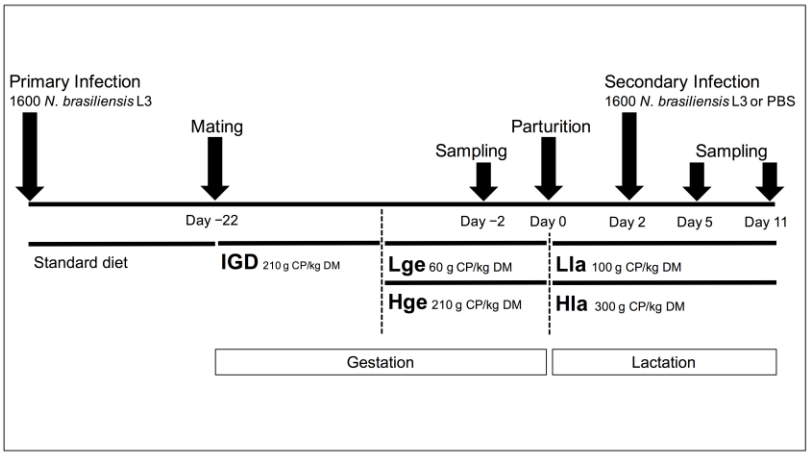

Primary Infection
1600 N. brasiliensis L3
Mating
Sampling
Parturition
Secondary Infection
1600 N. brasiliensis L3 or PBS
Sampling
Day −22
Day −2
Day 0
Day 2
Day 5
Day 11
Standard diet
IGD 210 g CP/kg DM
Lge 60 g CP/kg DM
Lla 100 g CP/kg DM
Hge 210 g CP/kg DM
Hla 300 g CP/kg DM
Gestation
Lactation

Figure 2

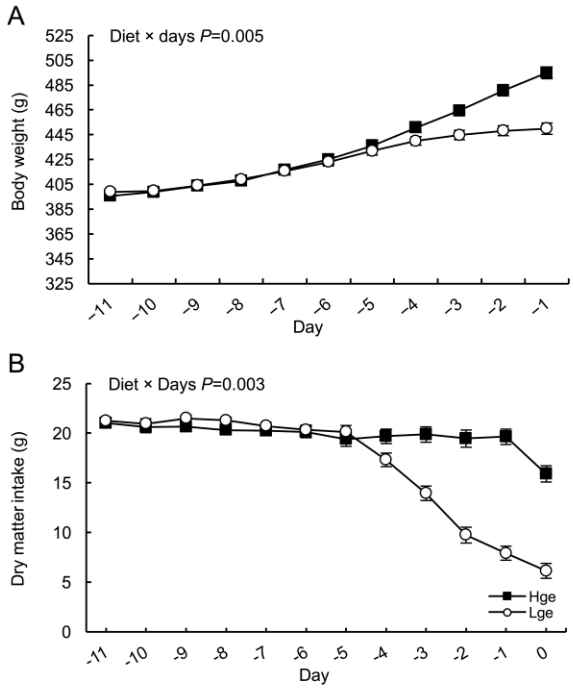

A
Diet × days P=0.005
Body weight (g)
525
505
485
465
445
425
405
385
365
345
325
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
Day
B
Diet × Days P=0.003
Dry matter intake (g)
25
20
15
10
5
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
Day
Hge
Lge

Figure 3

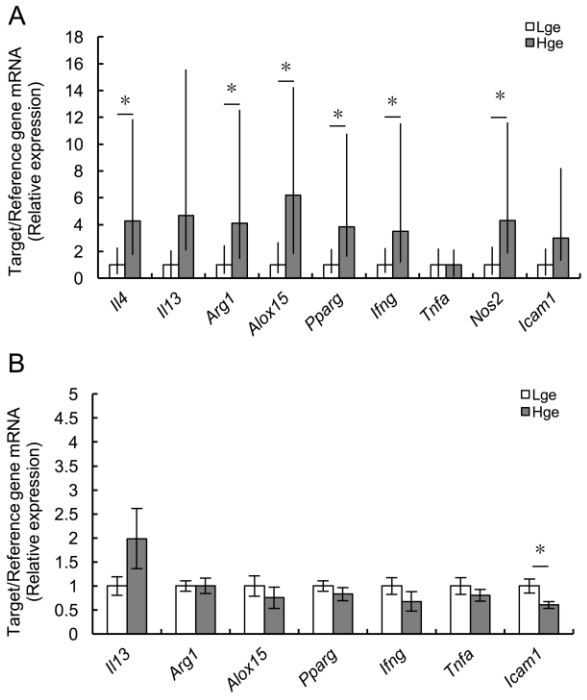

A
Lge
Hge
Target/Reference gene mRNA (Relative expression)
18
16
14
12
10
8
6
4
2
0
*
Il4
Il13
Arg1
Alox15
Pparg
Ifng
Tnfa
Nos2
Icam1
B
Lge
Hge
Target/Reference gene mRNA (Relative expression)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Il13
Arg1
Alox15
Pparg
Ifng
Tnfa
Icam1

Figure 4

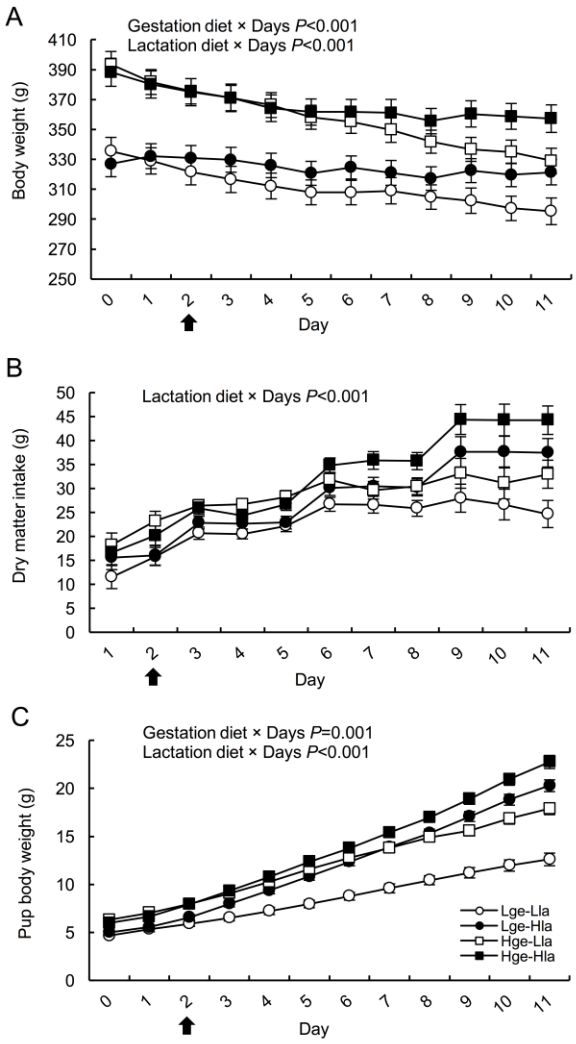

A
Gestation diet × Days P<0.001
Lactation diet × Days P<0.001
Body weight (g)
410
390
370
350
330
310
290
270
250
0 1 2 3 4 5 6 7 8 9 10 11
Day
B
Lactation diet × Days P<0.001
Dry matter intake (g)
50
45
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11
Day
C
Gestation diet × Days P=0.001
Lactation diet × Days P<0.001
Pup body weight (g)
25
20
15
10
5
0
Lge-Lla
Lge-Hla
Hge-Lla
Hge-Hla
0 1 2 3 4 5 6 7 8 9 10 11
Day

Figure 5

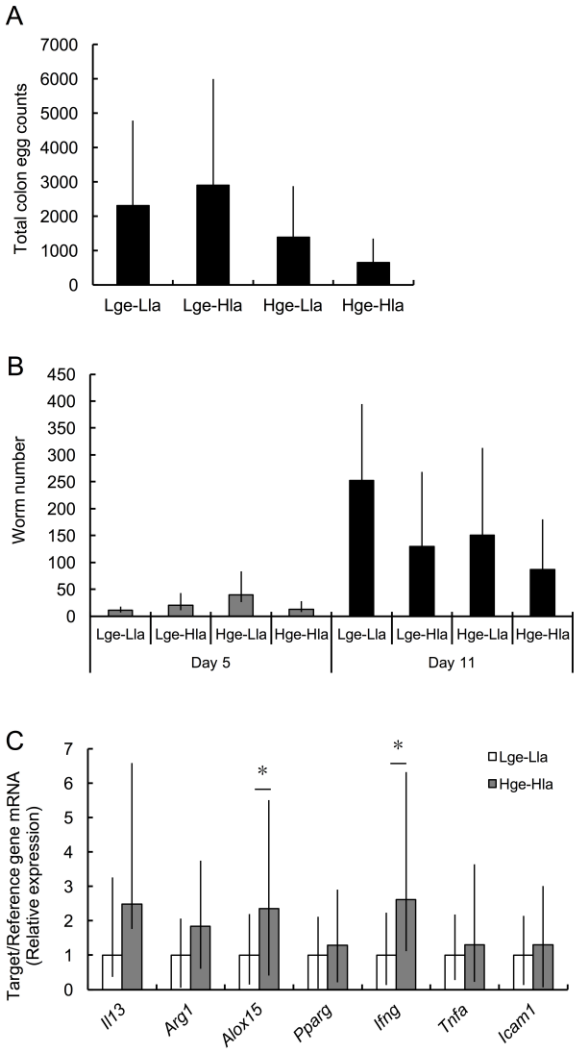

A
Total colon egg counts
7000
6000
5000
4000
3000
2000
1000
0
Lge-Lla
Lge-Hla
Hge-Lla
Hge-Hla
B
Worm number
450
400
350
300
250
200
150
100
50
0
Lge-Lla
Lge-Hla
Hge-Lla
Hge-Hla
Lge-Lla
Lge-Hla
Hge-Lla
Hge-Hla
Day 5
Day 11
C
Target/Reference gene mRNA
(Relative expression)
7
6
5
4
3
2
1
0
*
*
Lge-Lla
Hge-Hla
Il13
Arg1
Alox15
Pparg
Ifng
Tnfa
Icam1

Figure 6

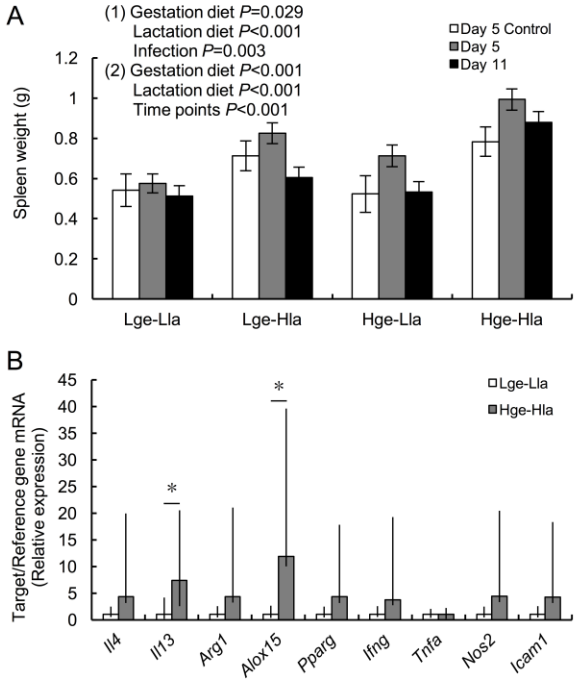
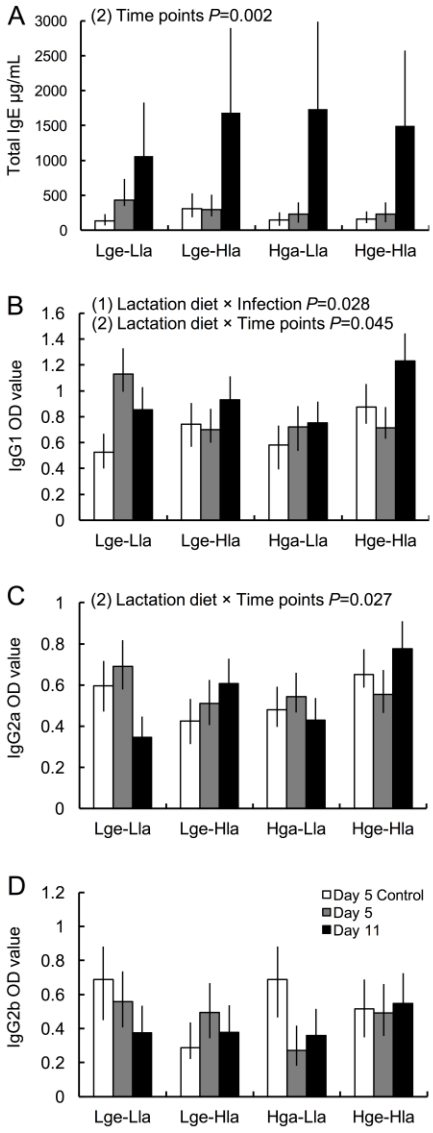

A
(1) Gestation diet P=0.029
Lactation diet P<0.001
Infection P=0.003
(2) Gestation diet P<0.001
Lactation diet P<0.001
Time points P<0.001
Day 5 Control
Day 5
Day 11
Spleen weight (g)
1.2
1
0.8
0.6
0.4
0.2
0
Lge-Lla
Lge-Hla
Hge-Lla
Hge-Hla
B
Lge-Lla
Hge-Hla
Target/Reference gene mRNA (Relative expression)
45
40
35
30
25
20
15
10
5
0
*
*
Il4
Il13
Arg1
Alox15
Pparg
Ifng
Tnfa
Nos2
Icam1

Figure 7


A
(2) Time points P=0.002
Total IgE μg/mL
3000
2500
2000
1500
1000
500
0
Lge-Lla
Lge-Hla
Hga-Lla
Hge-Hla
B
(1) Lactation diet × Infection P=0.028
(2) Lactation diet × Time points P=0.045
IgG1 OD value
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Lge-Lla
Lge-Hla
Hga-Lla
Hge-Hla
C
(2) Lactation diet × Time points P=0.027
IgG2a OD value
1
0.8
0.6
0.4
0.2
0
Lge-Lla
Lge-Hla
Hga-Lla
Hge-Hla
D
Day 5 Control
Day 5
Day 11
IgG2b OD value
1.2
1
0.8
0.6
0.4
0.2
0
Lge-Lla
Lge-Hla
Hga-Lla
Hge-Hla